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PRINCIPAL INVESTIGATOR: Robert E. Johnston, Ph.D.  
Nancy L. Davis, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina  
at Chapel Hill

Chapel Hill, NC 27599-7290

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## INTRODUCTION

A full-length cDNA clone of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus (VEE) is the starting point for a molecular genetic approach to a new live, attenuated human-use vaccine for VEE (Davis *et al.*, 1989, 1991). RNA genome equivalents can be produced by *in vitro* transcription of this clone (pV3000) and transfected into susceptible cultured cells to begin a complete replicative cycle. The progeny virus produced (V3000) shows the growth properties and virulence phenotype of the TRD strain of VEE in all systems examined. The application of recombinant DNA techniques to combine multiple, independently attenuating mutations into a single virus genome, judging from long experience with live, attenuated vaccines for other viral pathogens, will provide a safe vaccine strain with a very low theoretical rate of reversion to a more virulent phenotype. Also, mutations can be selected that have minimal effects on immunogenicity.

Site-directed mutagenesis of the V3000 clone was used to identify single site attenuating mutations that significantly reduced virulence in mice (Davis *et al.*, 1991). Multiply attenuated clones were produced by successive rounds of mutagenesis, and shown to be further reduced in virulence using sensitive rodent models.

One class of mutant cDNA clones contained lethal mutations in the cleavage signal for the PE2 glycoprotein precursor, either a deletion of the entire four codon signal, or a nonconservative change in the fourth position of the signal (Annual Reports, 1993, 1994; Davis *et al.*, 1995). Although RNA transcripts from such clones were nonviable, viable revertants were isolated from transfected cultures. Second site mutations were identified in these revertants and shown to be responsible for suppressing the lethal PE2 cleavage site mutations. In addition, these viable double mutants were highly attenuated in rodent models (Davis *et al.*, 1995). Most of the

results presented in this report concern characterization of two of these PE2-noncleaving mutants.

Parallel testing of single and multiple attenuated mutants in sensitive animal models showed that multiple mutants do, in fact, give lower mortality at high doses. However, it has not been shown directly whether this reflects a different pathogenetic phenotype, or a lower rate of reversion *in vivo*. A reconstitution experiment with virulent and attenuated VEE strains is being used to design a protocol for quantitation of virulent revertants in vaccinated animals.

VEE can be transmitted by aerosol and intranasal routes (Jahrling and Stephenson, 1984; Kinney *et al*, 1988), and although these routes are not known to be part of the natural cycle of VEE transmission, they are an important consideration for laboratory workers and troops subject to attack by VEE as a biological weapon. Use of the current investigational vaccine, TC-83, dramatically reduced laboratory infections with VEE, and protected mice against intranasal challenge (Jahrling and Stephenson, 1984; Kinney *et al*, 1988). As reported previously (Annual Reports, 1993, 1994), subcutaneous immunization with molecularly cloned attenuated mutants conferred complete protection against a high dose intranasal challenge with V3000. We have examined the strength and extent of this protection using *in situ* hybridization to assay replication of the challenge virus.

## RESULTS

### I. Analysis of PE2-cleavage defective molecularly cloned VEE mutants

As reported previously (Annual Reports, 1993, 1994), full-length VEE cDNA clones with altered PE2 cleavage sites were constructed in the V3000 genetic background, either with or without a second suppressor mutation at E1 position 253. The suppressor mutation restored viability

to the RNA transcripts (Table 1). The E1 253 mutation was also placed alone into the V3000 background.

**TABLE 1**

**PROPERTIES OF MOLECULARLY CLONED PE2 CLEAVAGE SITE MUTANTS**

Clone	Sequence at PE2 cleavage Site <sup>a</sup>	Residue at E1 253 <sup>a</sup>	Viability of RNA Transcript <sup>b</sup>	Relative Specific Infectivity of Progeny Virus <sup>c</sup>
V3000	RKRR/S	Phe	+	1.0
V3022	----/S	Phe	-	0.0
V3038	RKRD/S	Phe	-	0.008
V3526	----/S	Ser	+	1.2
V3528	RKRD/S	Ser	+	1.2
V3040	RKRR/S	Ser	+	3.2

<sup>a</sup>Indicated mutations were placed in the full-length virulent clone, V3000, by replacement of mutagenized restriction fragments including either the E1 gene, the E2 gene or both.

<sup>b</sup>*In vitro* RNA transcripts were quantitated by radiolabeling with <sup>35</sup>S-UTP and used with Lipofectin (BRL) to transfect BHK cell monolayers, which were then overlaid with agarose to produce RNA-initiated plaques. Viability was assessed by comparing plaque forming units (pfu)/counts per minute (cpm) to a V3000 transcript control. + indicates specific infectivity between 10% and 100% of that measured in parallel for a viable control transcript, - indicates specific infectivity < 1% of viable control.

<sup>c</sup>BHK cell monolayers were either transfected with RNA transcripts (V3000, V3022, V3038, and V3040) or infected with transfection supernatants (V3000, V3526 and V3528) in the presence of <sup>35</sup>S-Met. Radiolabeled virions were purified and assayed for pfu and cpm. Values for pfu/cpm were calculated relative to V3000, set at 1.0, after correction for the Met content of E3. V3022, V3038 and V3040 were compared to V3000 in a single electroporation experiment. Values (relative to a V3000 control) for V3526 and V3528 represent an average of two separate infections.

Specific infectivity of PE2-containing particles

Plaques formed on baby hamster kidney (BHK) cell monolayers transfected with RNA transcripts derived from pV3526 or pV3528 were significantly smaller than those formed following transfection with V3000 RNA. This raised the possibility that transfection with pV3526 or pV3528 RNA transcripts might produce a small proportion of infectious E2-containing particles, undetected by polyacrylamide gel analysis, while the majority PE2-containing particles remain nonviable. Therefore, the specific infectivities [plaque forming units (pfu)/<sup>35</sup>S methionine counts per minute (cpm)] of particles were measured (Table 1). Purified radiolabeled virions were produced by

transfection of BHK cells with RNA transcripts from pV3000, pV3022, pV3038 or pV3040, or by infection of BHK cells with V3000, V3526 or V3528. The ratio of infectious particles (pfu) to total particles (cpm) was zero for V3022 and near zero for V3038, indicating that reversion, either second site or same site (for V3038), of these lethal cleavage site mutations is very low. The specific infectivities of the viable mutants were comparable to that of V3000, indicating that the population of mutant PE2-containing particles contained the same proportion of infectious virus as the parental E2-containing population. As alphaviruses are known to have low particle/pfu ratios [A ratio of 16 was measured previously for Sindbis virus using quantitative electron microscopy with a T4 phage internal control (D. C. Flynn and R. E. Johnston, unpublished results)], these results show that the PE2-containing viruses are infectious, and that phenotypes measured for this virus population are attributable to viruses containing unprocessed PE2. In addition, the suppressing mutation in E1 restored normal levels of infectivity to both types of noncleaving mutant, although it was originally isolated in conjunction with the deletion of the PE2 cleavage signal.

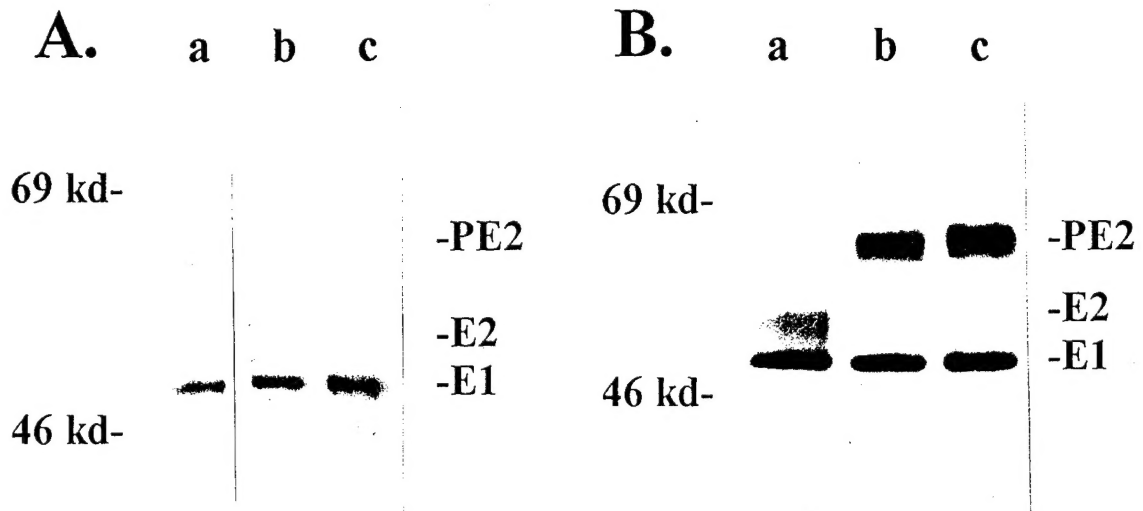
#### Low reversion rates of V3022 and V3038

As shown in Table 1, purified V3022 virions gave no pfu (limit of detection 250 pfu) under conditions where V3000 gave  $1 \times 10^7$  pfu/ml. V3038 gave 16 plaques at the  $10^{-2}$  dilution, and a very low specific infectivity compared to V3000. The 16 plaques were of various sizes, suggesting the presence of different resuscitating mutations. Thus, the rate of reversion of lethal cleavage signal mutations is very low, and the deletion appears less likely to be resuscitated than the substitution.

#### Polypeptide composition of viable and non-viable PE2-containing particles

Analysis of  $^{35}\text{S}$ -methionine labeled virion proteins by SDS-containing polyacrylamide

FIGURE 1

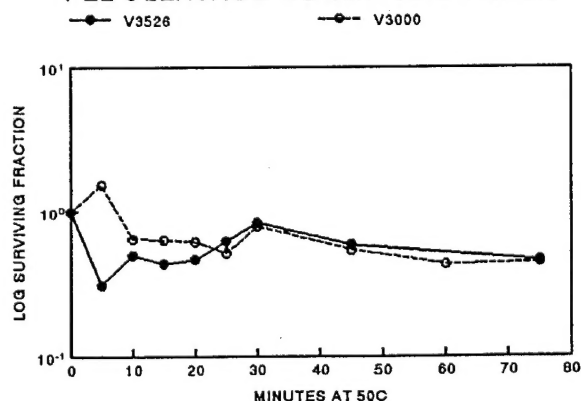


gel electrophoresis showed that the elimination or mutation of the highly conserved PE2 cleavage signal did not prevent the production of progeny VEE particles. The mutant virions contained only unprocessed PE2 glycoprotein in place of E2 (Fig. 1). This was the case whether or not the viability of the particles had been restored by the second site mutation at E1 253. No mature E2 glycoprotein was detected in virus particles produced by BHK cells transfected with RNA transcripts from pV3022 (Fig. 1A) or pV3038 (data not shown), or by BHK cells infected with V3526 or V3528 (Fig. 1B). V3040 and V3000 virions contained comparable proportions of E2 and E1 and no detectable unprocessed PE2 (Fig. 1B). Therefore, processing of PE2 was not required for formation of progeny VEE particles, but in the absence of second-site suppressing mutations, PE2-containing particles were not infectious.

#### Heat stability of V3000 and V3526

Initial attempts to purify PE2-containing viruses by centrifugation on potassium tartrate gradients were hampered by the apparent instability of these virus particles in high salt. This preliminary finding suggested that they may be generally less resistant to environmental stress than their virulent parent. Therefore, V3000 and V3526 were tested in parallel for stability during incubation at 50C (Fig.2). The mutant and parent showed identical heat stability. The titers dropped only 50% after 75

FIGURE 2  
HEAT STABILITY OF VEE  
PE2 CLEAVAGE MUTANT AND PARENT



minutes at the high temperature. Therefore, the structural alterations caused by the presence of PE2 instead of E2 in the virion must affect only some environmental conditions, eg. high salt, rather than reducing stability in general.

#### Attenuation of V3526 in CD-1 mice

Attenuation in animal models has been a consistent feature of PE2-containing alphavirus mutants (Russell *et al.*, 1989; Glasgow *et al.*, 1991; Heidner *et al.*, 1994; 1994a). Results obtained with V3526 and V3528 extend this observation to PE2-containing mutants of VEE. The data shown in Table 2 confirm results obtained with these mutants by our collaborators at

**TABLE 2**

#### **MOUSE VIRULENCE PHENOTYPES OF PE2-CONTAINING VEE MUTANTS**

Clone	Route of inoculation	Deaths/Total (average survival time)
V3000	subcutaneous <sup>a</sup>	4/4 (8.8 ± 0.75 days)
V3526	subcutaneous	0/8
V3526	intracerebral <sup>b</sup>	1/8 (8 days) <sup>c</sup>
V3528	subcutaneous	0/8
V3040	subcutaneous	1/8 (9 days)

<sup>a</sup>10<sup>3</sup> pfu were inoculated sc. into the left rear footpad in a volume of 10 µl.

<sup>b</sup>10<sup>3</sup> pfu were inoculated ic. into the left hemisphere near the midline in a volume of 10 µl.

<sup>c</sup>In similar experiments with V3000, mortality following ic. inoculation of 10<sup>3</sup> pfu was 100% with 7.0 ± 0.0 days average survival time.

USAMRIID in other mouse strains by other routes of infection, and in hamsters. Both V3526 and V3528 were avirulent, with no disease signs, when 10<sup>3</sup> pfu were inoculated into the left rear footpads of CD-1 mice sc. under conditions that reproducibly gave 100% mortality with V3000. V3526 was also significantly attenuated when introduced directly into the brain at a dose (10<sup>3</sup> pfu) that for V3000 routinely gives 100% mortality with an average survival time of 7.0 ± 0.0 days. In a separate experiment, no mortality or disease signs were observed in 6 of 6 mice at an intracerebral (ic.) dose

of  $10^3$  pfu of V3526, and at a dose of  $10^5$  pfu delivered i.c., 6/6 mice became ruffled and one died with signs typical of virulent VEE infection. Relative to several other molecularly cloned attenuated viruses, including both single and multiple mutants (Davis *et al.*, 1991), these PE2-containing mutants appeared to be the most highly attenuated in adult mice. The attenuation observed with V3040, containing the E1 Ser 253 mutation alone, first observed by collaborators at USAMRIID, was unpredicted but significant. The immunogenicity of all of these mutants was demonstrated by the resistance of immunized mice to intraperitoneal (ip.) challenge with a dose of V3000 ( $10^4$  pfu) that gave 100% mortality with an average survival time of  $5.5 \pm 2.5$  days in PBS-immunized controls.

V3526 also has been tested in monkeys at USAMRIID, using a single subcutaneous (sc.) immunization and challenging by aerosol with a high dose of V3000. The V3526-immunized animals were protected against V3000-induced disease.

#### Growth of V3526 in cultured cells

Our previous studies of V3526 suggested that this mutant was genetically unstable, in that variants with different plaque morphologies were produced during replication in BHK cells (Semi-annual report, Jan. 1995; Davis *et al.*, 1995). Three plaque isolates of V3526 grown on BHK cells, one large, one small and round, and one small and irregular (like the V3526 parent) have been tested in preliminary experiments for virulence in CD-1 mice and for growth on BHK cells. CD-1 mice were inoculated with  $10^3$  pfu of each plaque variant (four mice per variant) sc. in the left rear footpad. No deaths or clinical disease resulted during the 14 day observation period, and all mice were protected against ip. challenge with  $10^4$  pfu of virulent V3000. These variants appeared during growth on BHK cells, suggesting that they may enjoy a selective advantage in these cells. However, one further cycle of growth on BHK cells gave low titers (approximately  $5 \times 10^5$  pfu/ml) for all three

variants. Therefore, although plaque variants did arise from growth of V3526 on BHK cells, the results of this experiment do not indicate that the variants have increased virulence in CD-1 mice, or improved growth properties in BHK cells.

It was observed in previous studies with V3526 and with other molecularly cloned attenuated mutants, that serial passage in BHK cells led to progressively higher maximum virus titers. We have done a careful passage study of V3526 in BHK cells to document how many cycles of infection are required for this effect, and to form the basis for experiments designed to determine its cause. The starting material was a V3526 electroporation supernatant from BHK cells containing  $1 \times 10^7$  pfu/ml with a uniform small plaque morphology. BHK cell monolayers were infected at the indicated m.o.i. and supernatants were harvested at 24 hours. Titers were determined by plaque assay on BHK cells.

**TABLE 3**

**SERIAL PASSAGE OF V3526 IN BHK CELLS**

Sample	m.o.i.	virus titer (pfu/ml)	plaque size
electroporation supernatant		$1.0 \times 10^7$	uniform, 1/4xnormal size
P1	0.35	$1.6 \times 10^7$	10% 1/2xnormal, 90% 1/4xnormal
P2	2	$2.2 \times 10^8$	10% 1/2xnormal to normal, 90% 1/4normal
P3	6	$3.4 \times 10^8$	10% 1/2xnormal, 90% 1/4 normal

Because of the necessarily low m.o.i. in the first passage, this passage probably represents two cycles of replication. Therefore, in this experiment, an abrupt 10-fold increase in viral titer occurred between the second and third pass in BHK cells. Although large plaque variants did appear in the first low m.o.i. pass, they were not amplified in subsequent passes, and they were generally not as large as V3000 plaques. One possibility is that a mutant carrying a coding change in a viral protein was selected that makes either a more efficient RNA replicase/transcriptase, or an altered structural protein that is more efficient in attachment, penetration, uncoating, assembly and/or

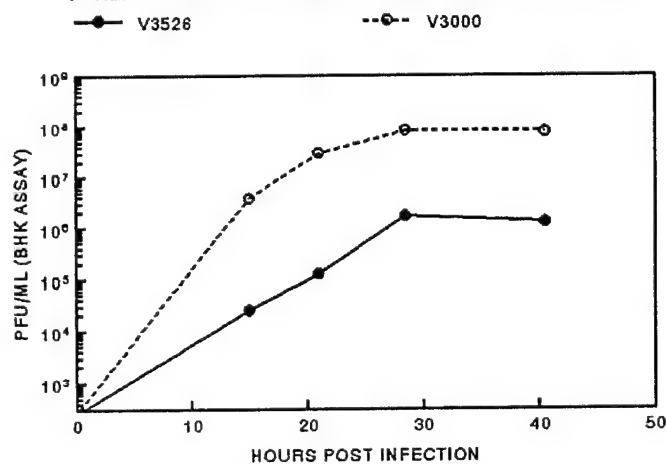
budding. Sequence analysis of virus produced by serial passage in BHK cells would determine whether this has occurred. Alternatively, a virus mutant may have been selected with a poly(A) tract longer than the 21 residues in the pV3526 clone, which may improve either the efficiency of viral RNA replication or translation, or the stability of intracellular progeny genomes. There is a precedent for this effect in the case of EMC virus, a picornavirus. Stocks of EMC were divided by oligo(dT) chromatography into three subpopulations with average poly(A) tract lengths of 16, 26 and 74 residues. RNAs with the longest poly(A) tracts showed a 10-fold higher specific infectivity in transfection experiments than those with the shortest tracts (Hruby and Roberts, 1976). As poly(A) tracts in alphavirus virion RNA are of variable lengths (Johnston and Bose, 1972), the replicase is believed to stutter as it copies this sequence. If such stuttering were to occur on transfected *in vitro* transcripts, and a longer poly(A) tract gave a replicative advantage, selection of a variant with this attribute might occur. Analysis of the specific infectivity of viral RNAs isolated from different passages and correlation with length of poly(A) tracts would demonstrate whether this is in fact the case.

The fact that the biological homolog of V3526 (J9-1) was first isolated on primary chicken embryo fibroblasts (CEFs) (Annual Report, 1993), coupled with the observation that electroporation stocks of V3526 made with BHK cells consistently contained only  $1 \times 10^7$  pfu/ml, raised the possibility that V3526 was adapted to growth on CEFs and grew relative poorly in BHK cells. To determine whether CEFs are more permissive than BHK cells for V3526, and would give higher titer stocks with less selective pressure for variation, CEFs were transfected by electroporation with RNA transcripts from pV3526. Transfection supernatants produced with two different voltages both contained viable progeny virus. The plaques given by the supernatants from electroporated cells were small and uniform, but the titers were approximately the same or slightly less than those

obtained from transfected BHK cells, about  $1-5 \times 10^6$  pfu/ml for both conditions. These results suggested that CEFs may not be a more permissive cell type for this mutant.

Two further experiments were done to determine the ability of CEFs to replicate V3526. CEFs were transfected by electroporation with RNA transcripts of either pV3000 or pV3526 and virus growth was assayed over time (Fig. 3). V3526 grew more slowly than V3000 and the peak titer of V3526 was 100-fold lower than V3000. In a second experiment, parallel cultures of BHK and CEF cells were infected with V3000 and V3526 at an m.o.i. of 1 pfu/cell and virus growth was assayed. By 20 hr post infection of

FIGURE 3  
GROWTH IN ELECTROPORATED CEFs  
PE2 CLEAVAGE MUTANT AND PARENT



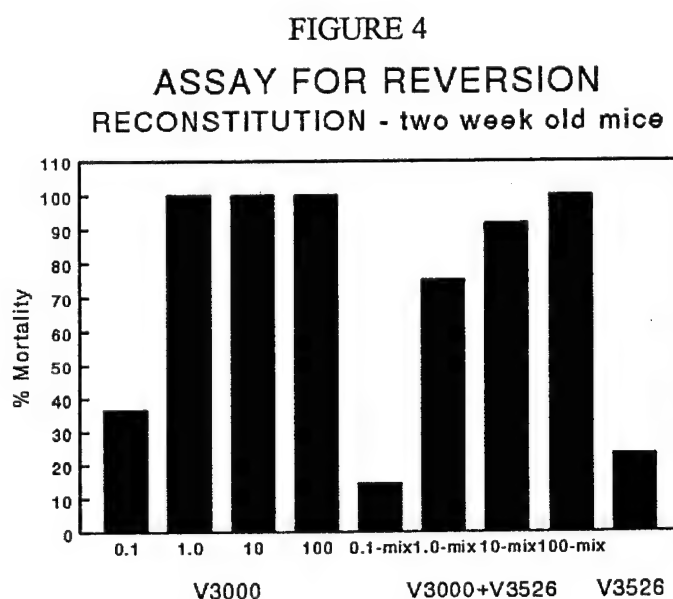
BHK cells, V3526 showed a titer of  $1 \times 10^8$  pfu/ml compared to  $7.8 \times 10^8$  pfu/ml for V3000. CEFs, however, gave lower titers of both V3000 and V3526,  $2.2 \times 10^8$  and  $2.2 \times 10^6$  pfu/ml, respectively. Therefore, it does not appear that the resuscitating mutation, E1 ser 253, is specific for growth in CEF, and may in fact give more efficient growth in BHK cells than in CEFs.

## II. Assay for *in vivo* reversion of attenuated mutants

The informed design of a safe and effective molecularly cloned VEE vaccine is based on the premise that combining multiple independently attenuating mutations will lower the rate of reversion in the vaccinee. In very sensitive animal models we showed previously that multiply

attenuated mutants gave lower mortality than single mutants (Davis *et al.*, 1991), but direct measurement of reversion *in vivo* is difficult. One approach would be to biologically clone viruses from tissues of mice infected with vaccine candidates, and test each virus clone for virulence phenotype in naive mice. An alternative to this time consuming protocol is to design a test in which virus-containing samples which may include a low proportion of virulent revertants, eg. tissue homogenates from vaccinated mice, are inoculated into animals and scored for morbidity and mortality. The fact that only a single plaque forming unit of virulent VEE is lethal for mice makes this type of test feasible. We have begun testing protocols for such an assay using mixtures of highly attenuated V3526 with varying amounts of virulent V3000. Adult CD-1 mice were inoculated ip. with  $10^5$  pfu of V3526 mixed with varying amounts of V3000 (from 0.1 pfu to 1000 pfu). In controls receiving only V3000, all inoculated mice died at a dose of 1 pfu or above. However, mice receiving the mixed viruses showed 16% mortality at 1 V3000 pfu, 0% at 10 pfu, 30% at 100 pfu and 16% at 1000 pfu.  $10^5$  pfu of V3526 alone gave 0% mortality. This first experiment showed that in the adult mouse, the presence of an otherwise lethal amount of V3000 when mixed with  $10^5$  pfu of V3526 did not consistently result in death.

Therefore, the same experiment was carried out in more sensitive two week old CD-1 mice (Fig. 4). In this test, even 0.1 pfu of V3000 alone gave 35% mortality, with all higher doses resulting in 100% mortality.  $10^5$  pfu of V3526 alone gave 25% mortality. However,



mixing only 1 pfu of V3000 with  $10^5$  pfu of V3526 resulted in 75% mortality, within the range that would be predicted for a 1 pfu dose of V3000 alone. Higher amounts of V3000 produced 95% and 100% mortality. Thus, in two week old mice, a reversion frequency of  $10^{-5}$  could be detected. We are now studying possible effects of cytokines present in tissue homogenates on the infectivity of virus tested in this way. If this source of possible interference can be eliminated, ip. inoculation of serial dilutions of clarified tissue homogenates from vaccinated mice into two week old CD-1 mice will provide a sensitive screen for the rate of reversion to a virulent phenotype.

### **III. Protection against intranasal challenge with virulent VEE**

We reported previously that a single sc. inoculation into the left rear footpad of CD-1 mice with the attenuated mutant, V3014 (E2lys209, E1thr272) gave complete protection against disease following intranasal (in.) challenge with a high dose of virulent V3000 (Annual Report, 1993). When the footpad, draining popliteal lymph node, spleen, olfactory neuroepithelium, olfactory bulb, brain and serum of challenged animals were assayed for the presence of viable virus, none was found at any of several time points between 6 and 24 hr post-challenge (Annual Report, 1994). It was possible that the high levels of anti-VEE serum antibody in these mice at the time of challenge may have masked a low level of replication of challenge virus. Therefore, *in situ* hybridization (as described in Grieder *et al.*, 1995) with a VEE-specific riboprobe was used to assay for challenge virus replication. Tissues from nasal neuroepithelium, olfactory bulb and other CNS structures were assayed at 24 and 72 hrs after in. challenge with V3000. Tissues from V3000-challenged, mock-vaccinated mice showed large amounts of newly synthesized V3000 RNA, coupled with extensive pathology throughout the full thickness of the olfactory neuroepithelium and invasion of the CNS. However, in V3014-vaccinated mice no VEE RNA was detected. These data strongly suggested that

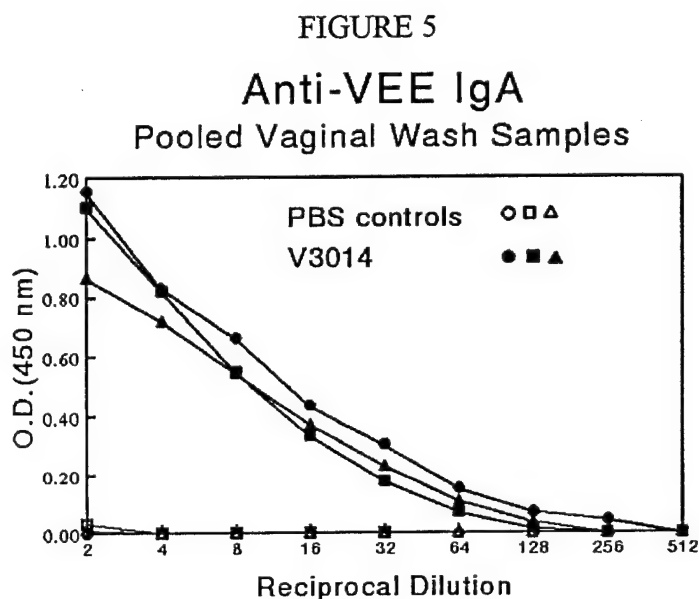
the apparently complete protection against virulent in. challenge was mediated at the level of the nasal mucosa itself.

Anti-viral IgA has been implicated in protection of the nasal mucosa of mice against invasion by influenza virus and Sendai virus (Mazanec *et al.*, 1987; Renegar and Small, 1991). Therefore, V3014-inoculated mice were assayed for VEE-specific mucosal IgA. Pooled and

concentrated vaginal washes taken at 10, 14 and 21 days post immunization were used as the source of antibody in an ELISA with purified VEE virus antigen and goat anti-mouse IgA second antibody (Fig. 5). Significant levels of VEE-specific IgA were measured relative to unvaccinated controls. Delineating the mechanism by which a subcutaneous inoculation of attenuated VEE induces mucosal immunity may broaden our understanding of the mucosal immune system and the interaction between attenuated VEE vaccine strains and the host.

## CONCLUSIONS

Further characterization of PE2-cleavage defective attenuated mutants of VEE has confirmed the strong attenuating effect of these mutations in mice. Even a dose of  $10^5$  pfu of V3526



delivered directly into the brain gave signs of fever, but no neurological effects, in 84% of inoculated animals with only 16% mortality. Specific infectivity measurements showed that the presence of PE2 rather than mature E2 in the virion is the cause of the attenuated phenotype of these mutants. The presence of PE2 in these particles did not make them less heat stable than their V3000 parent. The growth rate in cultured cells was examined further to determine whether the second-site suppressor mutation of the lethal PE2-cleavage site mutations, originally selected on CEFs, was cell type specific. This was not the case, since replication of these mutants seemed more efficient in BHK cells than CEFs. Serial passage of V3526 in BHK cells, however, did result in an abrupt increase in peak titer after three passes. This did not, in this experiment, correlate with overgrowth of a plaque variant, but may reflect selection of a BHK-adapted mutant or variant with a longer poly(A) tract.

Results from a reconstitution experiment indicate that a reversion frequency as low as  $10^{-5}$  to a virulent phenotype could be detected in a population of V3526. Therefore, it may be possible to screen tissue homogenates from vaccinated mice for the presence of virulent revertants.

The strong protection conferred by a single sc. dose of the doubly attenuated mutant V3014 (E2lys209, E1thr272) against in. challenge with virulent V3000 was studied using an *in situ* hybridization assay for challenge virus replication. The single parenteral dose of an attenuated VEE mutant appeared to result in complete resistance of the nasal mucosa to virus invasion. Measurement of high levels of anti-VEE vaginal IgA in V3014-vaccinated mice suggested that the secretion of neutralizing VEE-specific IgA antibody onto the nasal mucosa might be the mechanism for protection.

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## PUBLICATIONS AND ABSTRACTS

These titles represent all the papers and meeting abstracts concerning VEE produced by our lab and by our collaborators during the past year. They describe work supported in whole or in part by this contract.

- Davis, N. L., Brown, K. W., Greenwald, G. F., Zajac, A. J., Zacny, V. L., Smith, J. F., and Johnston, R. E. (1995). Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second site suppressor mutation in E1. *Virology*, in press.
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